



# Cancer Stem Cells—Origins and Biomarkers: Perspectives for Targeted Personalized Therapies

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Walcher L, Kistenmacher A-K, Suo H, Kitte R, Dluczek S, Strauß A, Blaudszun A-R, Yevsa T, Fricke S and Kossatz-Boehlert U (2020) Cancer Stem Cells—Origins and Biomarkers: Perspectives for Targeted Personalized Therapies. Front. Immunol. 11:1280. doi: 10.3389/fimmu.2020.01280 The use of biomarkers in diagnosis, therapy and prognosis has gained increasing interest over the last decades. In particular, the analysis of biomarkers in cancer patients within the pre- and post-therapeutic period is required to identify several types of cells, which carry a risk for a disease progression and subsequent post-therapeutic relapse. Cancer stem cells (CSCs) are a subpopulation of tumor cells that can drive tumor initiation and can cause relapses. At the time point of tumor initiation, CSCs originate from either differentiated cells or adult tissue resident stem cells. Due to their importance, several biomarkers that characterize CSCs have been identified and correlated to diagnosis, therapy and prognosis. However, CSCs have been shown to display a high plasticity, which changes their phenotypic and functional appearance. Such changes are induced by chemo- and radiotherapeutics as well as senescent tumor cells, which cause alterations in the tumor microenvironment. Induction of senescence causes tumor shrinkage by modulating an anti-tumorigenic environment in which tumor cells undergo growth arrest and immune cells are attracted. Besides these positive effects after therapy, senescence can also have negative effects displayed post-therapeutically. These unfavorable effects can directly promote cancer stemness by increasing CSC plasticity phenotypes, by activating stemness pathways in non-CSCs, as well as by promoting senescence escape and subsequent activation of stemness pathways. At the end, all these effects can lead to tumor relapse and metastasis. This review provides an overview of the most frequently used CSC markers and their implementation as biomarkers by focussing on deadliest solid (lung, stomach, liver, breast and colorectal cancers) and hematological (acute myeloid leukemia, chronic myeloid leukemia) cancers. Furthermore, it gives examples on how the CSC markers might be influenced by therapeutics, such as chemo- and radiotherapy, and the tumor microenvironment. It points out, that it is crucial to identify and monitor residual CSCs, senescent tumor cells, and the pro-tumorigenic senescence-associated secretory phenotype in a therapy follow-up using specific biomarkers. As a future perspective, a targeted immune-mediated strategy using chimeric antigen receptor based approaches for the removal of remaining chemotherapy-resistant cells as well as CSCs in a personalized therapeutic approach are discussed.

Keywords: cancer stem cells, senescence, targeted therapy, CAR cells, biomarkers, precision therapy

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# INTRODUCTION

In 2018, according to the GLOBOCAN study, the malignant neoplasms with the highest mortality were lung (1.76 million deaths), stomach (783,000 deaths), liver (782,000 deaths), breast (627,000 deaths), and colorectal cancers (551,000 deaths) as well as blood cancers including leukemia (309,000 deaths) (1). All of these cancers are heterogeneous tumors containing cells with various stem cell properties, as described below. Already in 1877, Virchow's student Cohnheim noticed this cell population and pointed out that it possesses an embryonic character (2). Today, those cells are called cancer stem cells (CSCs) or tumor-initiating cells (TICs) and are seen as drivers of tumor establishment and growth (2-5), often correlated to aggressive, heterogeneous and therapy-resistant tumors (6, 7). Upon application of therapeutic regimens such as chemo- or radiotherapy the composition of tumor cell subpopulations changes (6, 8). At first, tumor cells with a high proliferative capacity are targeted and depleted causing a decrease in tumor size while CSCs survive (9). Additionally, some tumor cells will become senescent [therapy-induced senescence (TIS)], and subsequently can cause a change in the tumor microenvironment (TME) with tumor promoting effects due to the senescenceassociated secretory phenotype (SASP) (6, 10-12).

It is well-known that CSCs are resistant to treatment and can cause tumor relapses (13). However, under the therapeutic pressure and changed microenvironment CSCs can be newly generated. In this case, these cells do originate from non-CSCs or from therapy-induced senescent tumor cells (14–18). It is therefore of importance to characterize these cells in detail and to understand their origin at the time of tumor initiation and tumor relapse.

This review underlines the role for a thorough investigation of tumors especially in the post-therapeutic period. Such posttherapeutic or therapy follow-up diagnostics are not conducted in the clinic on a regular basis, yet. The importance of specific biomarkers that analyze several parameters, such as CSCs phenotypes, senescence and TME composition, will allow the detection of therapy-resistant CSCs that cause tumor recurrence. A precise elimination of those cells of risk in a timely fashion using targeted cellular therapeutic approaches as the second line therapy is discussed in this study.

# CSCs AND THEIR ORIGIN AT TUMOR INITIATION

Tumor initiation can either be driven by transformed differentiated cells or transformed tissue resident stem cells (19) (compare **Figure 1**). The transformation can take place during tissue regeneration and can additionally, be initiated and/or accelerated as a response to infections, toxins, radiation or metabolic influences causing mutations (20, 21). During the transformation process, oncogenes are overexpressed and tumor suppressors are inactivated promoting uncontrolled growth of the cells (19). As a consequence, cells de-differentiate and acquire stem cell characteristics (19). The transformation of tissue

resident stem cells or their progeny is believed to presuppose a different set of genomic changes allowing uncontrolled, nicheindependent proliferation (5, 22). As stem cells already possess unlimited growth potential, it is believed that the transformation of stem cells and their progeny requires only few genomic changes (5, 22, 23). For example, the low mutagenic changes, identified in more than 10% of gastric cancers suggest that these tumors arise from tissue resident stem cell populations (24). Two stem cell populations have been identified in gastric cancers: slow cycling cells expressing the transcription factor Mist1 in the gastric corpus and Leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5)-expressing cells in the gastric antrum (25-27). Both populations have been linked to cancer generation in mouse models (24, 26, 27). In colon cancers, recent studies in mice have shown that even differentiated intestinal epithelial cells can be potential CSCs (28). The fact that adult differentiated cells, tissue resident stem cells or their progeny can promote tumor generation has also been shown in the liver. Cell tracking, in vitro and in vivo studies showed that liver cancer can originate from adult hepatocytes (29-32) as well as from hepatoblasts and hepatic progenitors (31, 32).

Tumor type, prognosis and aggressiveness are also influenced by the origin of the tumor, as analyzed for instance in breast cancers (33–35). Breast tumors originating from luminal progenitors are associated with a good prognosis, except those overexpressing Her2 (34, 36). Tumors originating from basal-like progenitors show a very aggressive phenotype (34).

In squamous cell carcinomas the differentiation phenotype seems to be influenced by the cell of origin and the kind of driver mutation, both responsible for the invasiveness and aggressiveness of the tumor (37, 38). Loss of the phosphatase and tensin homolog (Pten) as well as the liver kinase B1 (Lkb1) in lung epithelia causes tumor formation of highly penetrant tumors. These tumors are rarely metastatic and are characterized by a differentiated phenotype (37). Basal cells located within the trachea and main bronchi have been shown to self-renew and to form heterogeneous spheres (39). These basal stem cells can cause basal cell hyperplasia or epithelial hypoplasia, finally resulting in squamous cell metaplasia or dysplasia, which are discussed as precursors of squamous cell lung carcinomas (SCC) (39, 40). Studies by Fukui et al. suggest that high basal cell signatures correlate to a clinically aggressive phenotype in lung adenocarcinoma (40). Adenocarcinomas are considered to originate from sub-segmental airways of the bronchioalveolar stem cells or Type I and Type II pneumocytes (39). These bronchioalveolar stem cells are quiescent in healthy lungs but can enter proliferation cycles and could be targets of mutations causing transformation (39, 41). In mouse models, data indicate that small cell lung cancers (SCLC) can also originate from other cell types, i.e., neuroendocrine cells (42).

While in solid tumors the origin is heavily discussed, in hematological tumors the situation seems to be clearer. In acute myeloid leukemia (AML), the cell of origin is thought to be a hematopoietic stem or progenitor cell (43). However, a subgroup of human AML has been shown to share expression profiles with lymphoid T-cell progenitors. The authors showed that



under oncogenic conditions, DN2 (double negative 2) T-cell progenitors process into lymphoid, biphenotypic, and myeloid leukemia cells (43–45). In chronic myeloid leukemia (CML), the cell of origin is characterized by the expression of the Bcr-Abl oncogene, generated from a chromosomal translocation between chromosome 9 and 22 (46, 47). This molecular aberration defines the chronic phase in CML, which progresses into blast crisis upon additional mutations that promote self-renewal (46, 47). While leukemic stem cells (LSCs) are well-defined and characterized in AML and CML, the concept of CSCs in acute lymphoblastic leukemia (ALL) and also in non-hodgkin lymphoma (NHL) is less established (48–50).

Tumors generated on the basis of CSCs are believed to follow a unidirectional hierarchy, in which only the CSC population is able to initiate tumor growth (51). At the time point of tumor initiation, it is suggested that cancer stem cells divide asymmetrically to maintain the CSC pool (52). These asymmetric divisions generate transient amplifying cells, which are undergoing symmetric divisions; therefore having a high proliferative capacity (51, 52). Based on recent data from hematological cancers (AML), the hierarchical model proposed by Bonnet and Dick (43) is most likely a simplified description. It is now believed that the organization of CSCs (in solid as well as in hematological cancers) is more complex (52–56). In contrast to the CSC model in which only a small subpopulation of cells is able to promote tumor initiation and growth, the clonal evolution model states that genetically unstable cells accumulate genomic and genetic alterations over time causing an increase in tumor aggressiveness, resistance and heterogeneity (5, 57). Both models are not mutually exclusive, which can be explained by the cellular plasticity (plasticity model) that suggests, that different cellular states can interconvert (as explained later) (5, 57).

Because CSCs have been shown to cause tumor initiation and tumor relapses, the search for biomarkers that characterize these cells and allow therapeutic as well as prognostic prediction or follow-up is ongoing. The most prominent markers of solid and hematological tumors are described in the following section.

# Biomarkers for CSC Populations in Solid Cancers

In solid cancers, the clinical use of CSC specific biomarkers is very limited, besides the use of the carcinoembryonic antigen (CAE), fragments of the cytokeratin 19 (YFRA 21-1) (58) and the alpha-fetoprotein (AFP) that is expressed by cancer stem cells (58, 59). Importantly, most markers expressed in CSCs can also be found in adult tissue resident stem cell populations, human embryonic stem cells (hESC) or adult tissues (60). Additionally, most markers label heterogeneous stem cell populations pointing to the fact that their characterization and isolation has to be based on marker combinations using several surface markers or combinations of extracellular as well as intracellular markers; to

TABLE 1   Examples of lung cancer stem cell markers and their use as
diagnostic, predictive, or therapeutic biomarkers.

Marker	Stem cell marker	Biomarker diagnostic	Biomarker therapeutic	Biomarker prognostic
Surface ma	arkers, CD			
CD44 (and its variants)	(61–66) (39, 67–69)* (70)**	(71)	(71–80)	(61, 64, 70, 81) (39, 69)*
CD87	(82)			
CD90	(83) (39, 67)*			
CD133	(84–99) (39, 67–69)* (70, 100)**		(74, 101–104) (69)*	(91, 105–112) (39, 67, 68)* (70)**
CD166	(62, 66, 113) (39, 68)*			(113)
Surface ma	arkers, not CD			
EpCAM	(62, 66, 86, 114, 115)	(116–120)	(121)	(117, 122–124)
Intracellula	r markers			
ALDH	(65, 84, 114, 125– 129) (39, 68, 69, 130)*	(131)	(132–134)	(62, 128, 135) (39, 69, 130)* (70, 126)**
Nanog	(70)			(70, 126) (69)*
Oct-3/4	(96) (67, 69)*		(67)*	(136) (69)*

The table lists examples of cancer stem cell markers and indicates those which have been tested as biomarkers within a therapeutic (metastasis, tumor stage, size), diagnostic, or prognostic (survival, resistance etc.) approach. Starsindicate reviews (\*) and contradictory results (\*\*).

identify and isolate cells that promote tumor initiation, resistance and relapse.

Below, a short summary of the most prominent markers is provided. CSC markers that could have potential usefulness within therapeutic, diagnostic, and prognostic approaches are pointed out (compare **Tables 1**–7) and focus on most deadliest tumors of lung, liver, breast, stomach, and colorectal as well as AML and CML. **Tables 1**–7 provide an extensive list of markers expressed in CSCs. A comparison shows that several markers are expressed in several tumor types.

### **CD44**

CD44 is a biomarker which is not only expressed in solid but also in hematological cancers (see below). Its expression is associated with increased proliferation, self-renewal and metastasis (61, 149, 462, 463). For example, in colorectal cancers, expression of CD44/CD166 characterizes a cell population able to form tumor spheres, suggesting anchorageindependent proliferation of these cells (333). In other studies, CD44<sup>high</sup>/CD133<sup>high</sup> cells showed increased tumorigenic capabilities (334). Also in breast cancers, the percentage of CD44<sup>+</sup>/CD24<sup>-</sup>/CK<sup>+</sup>/CD45<sup>-</sup> cells was shown to be increased in malignant lesions compared to non-malignant lesions (139). A significant decrease in proliferation and migration of breast cancer cells was observed after the knock-down of **TABLE 2** | Examples of breast cancer stem cell markers and their use as diagnostic, predictive, or therapeutic biomarkers.

Marker	Stem cell marker	Biomarker diagnostic	Biomarker therapeutic	Biomarker prognostic
Surface ma	arkers, CD			
CD24	(137)			
CD29 (ß1 integrin)	(137, 138)			
CD44 (and its variants)	(139–149)	(150–154)	(76, 150, 152, 154–166)	(166–171) (172, 173)**
CD49f	(174–176) (177)*		(178)	(175, 178, 179)
CD61	(137, 180)			
CD70				(181)
CD90	(182)			
CD133	(183) (184)*	(185–187)	(188–190) (184)*	(191–193) (184)*
Surface ma	arkers, not CD			
CXCR4	(194)			
EpCAM		(186)	(186)	
LGR5	(195)			(195)
ProC-R	(196)			
Intracellula	ar markers			
ALDH	(147, 148, 197, 198) (199, 200)*		(198, 201, 202) (199)*	(171, 192, 197, 203–208) (200)* (209, 210)**
BMI-1			(143, 211–218) (219)*	
Nanog			(142)	(220, 221)
Notch	(222–224)	(222, 225)	(187, 212, 222, 224, 226–230)	(222, 226, 231– 234) (235)*
Oct-3/4		(142)		(220, 221)
Sox2		(142)		
Signaling p	oathways			
Wnt/ß- Catenin	(195, 236, 237)	(236)	(237)	

The table lists examples of cancer stem cell markers and indicates those which have been tested as biomarkers within a therapeutic (metastasis, tumor stage, size), diagnostic, or prognostic (survival, resistance etc.) approach. Stars indicate reviews (\*) and contradictory results (\*\*).

CD44 (140). In gastric cancers, the knock-down of CD44 reduced sphere formation and caused decreased tumor growth in severe combined immunodeficiency mice (246). In many tumors (e.g., breast and liver), CD44 is expressed as isoform and its expression has been associated with increased cancer stem cell properties (141). In lung cancers, CD44v9 expression correlates significantly with early-stage lung adenocarcinoma and epidermal growth factor receptor (EGFR) mutations (464). Variants of CD44 are also expressed in gastric cancers and promote tumor initiation (248).

The CSC marker CD44 has been indicated as a biomarker for diagnostic, therapeutic, and prognostic approaches (compare **Tables 1–5**). In gastric cancer patients, CD44<sup>+</sup> circulating tumor cells correlated with a poor prognosis (465). In colorectal

<b>TABLE 3</b>   Examples of gastric cancer stem cell markers and their use as
diagnostic, predictive, or therapeutic biomarkers.

Marker	Stem cell marker	Biomarker diagnostic	Biomarker therapeutic	Biomarker prognostic
Surface ma	irkers, CD			
CD24	(238) (239)*	(240)*	(241)	(242–244) (239, 245)*
CD44 (and	(246-251)	<b>(</b> 247, 251,	(255–257)	(247, 251, 254,
its variants)	(239, 240, 245, 252)*	253, 254) (240)*	(239, 240, 245)*	258–260) (239, 240, 245, 252)*
CD90	(251) (239, 245)*			
CD133	(247, 249, 250) (239, 240, 252)*	(254, 261) (240)*	(257) (239, 240)*	(254, 262–265) (239, 240, 252)*
Surface ma	rkers, no CD			
CXCR4	(266)		(267)*	(268–271)
EpCAM	(248, 249, 272) (239, 240, 252)*		(273)	(265, 272)
LGR5	(274) (252)*	(240)*	(275, 276) (252)*	(275, 277–279)
LINGO2	(280)			(280)
Intracellula	r markers			
ALDH	(249, 281, 282) (239, 240, 252)*			(260, 281, 282)
Letm1	(283)			(283)
Musashi2	(284)			(284)
Nanog	(285) (239, 286)*	(287) (240)*		(287, 288) (286)*
Oct-3/4	(239, 252)* (289)**			(247, 265, 288) (289)**
Sox2	(247) (239, 240, 252, 290)*	(240)* (291)**	(292)	(247, 288, 293) (265)**

The table lists examples of cancer stem cell markers and indicates those which have been tested as biomarkers within a therapeutic (metastasis, tumor stage, size, resistance), diagnostic (i.e., resistance), or prognostic (survival, resistance etc.) approach. Stars indicate reviews (\*) and contradictory results (\*\*). **TABLE 4** | Examples of liver cancer stem cell markers and their use as diagnostic, predictive, or therapeutic biomarkers.

Marker	Stem cell marker	Biomarker diagnostic	Biomarker therapeutic	Biomarker prognostic
Surface ma	arkers, CD			
CD24	(294–296) (297, 298)*		(298)*	(295)
CD44	(299, 300) (298)*			(300–303) (298)* (304)**
CD90	(295, 300, 305–308) (297, 298)*			(295, 300, 304, 309) (298)*
CD133	(295, 296, 300, 310–313), (297, 298)*	<b>(</b> 314 <b>)</b>		(295, 300, 304, 311, 314– 319), (320)**, (298)*
Surface ma	arkers, not CD			
EpCAM	(297, 298)* (294, 300, 304, 311, 321)	(322)	(298)*	(300, 301, 304, 311, 319, 321– 327) (298)*
Intracellula	r markers and pat	hways		
AFP	(311, 321)		(328)	(311, 321, 329), (330)*
Nanog	(312, 313, 331), (298)*		(298)*	(331) (298)*
Notch	(295, 296, 305)		(295)	
Oct-3/4	(313, 331), (298)*			(309, 331), (298)*
Sox2	(313) (298)*			
Wnt/ ß-catenin	(295, 313)		(295)	(313) (330)*, **

The table lists examples of cancer stem cell markers and indicates those which have been tested as biomarkers within a therapeutic (metastasis, tumor stage, size, resistance), diagnostic (i.e., resistance), or prognostic (survival, resistance etc.) approach. Stars indicate reviews (\*) and contradictory results (\*\*).

cancers, a prognostic quantitative real-time PCR was established m to analyze the expression of CD44v2 showing that a high h expression correlated with a worse prognosis (339). In gastric e cancers, the expression of CD44 and CD90 correlated with th distant metastasis and could therefore be used as a diagnostic c biomarker (251) and was suggested as a biomarker for treatment response (253). Therapeutic approaches targeting CD44 have been made using e.g. adenoviral delivery of siRNA *in vitro* (337). Furthermore, CD44-targeting drug conjugated aptamers (76) or hyaluronic acid coated onto nanoparticles have been in the focus of research (155). Antibody-based photosensitizer conjugates for combined fluorescent detection and photo-immunotherapy (PIT) of CD44-expressing cells in triple-negative breast cancers (TNBC) (150) or other antibody-based approaches tested in safety studies (466–468).

### CD133

The biomarker CD133 (Prominin-1) is expressed on hESCs and rarely found on normal tissue cells (60). The marker has been additionally identified in tumors of breast, liver, stomach, and

colon (compare **Tables 1–5**) and has also been described as a marker that characterizes cells with high tumorigenicity and a high ability to form spheroids (184, 469). In breast cancers, its expression correlates with N-cadherin expression that was found to be significantly higher in patients with metastasis (191). In lung cancers, the expression of CD133 has been correlated to epithelial to mesenchymal transitions (EMT), in combination with other additional stem cell markers, such as BMI1 (84).

The expression of CD44 and CD133 in colorectal cancers can predict metastasis (470), however, no correlation to patient outcome could be detected (471). In breast cancers, CD133 mRNA was suggested to be suitable for prognosis prediction (193, 472) and CD133 protein has been correlated to a poor prognosis (193). Pre-clinical therapeutic approaches cover antibody-based targeting of colorectal (341, 342) as well as breast cancers (188) (compare **Tables 1–5**).

### EpCAM

The epithelial cell adhesion molecule (EpCAM, CD326) is expressed on CSCs in various tumor types including colon and hepatocellular cancers (473–476). Furthermore, it is expressed

Marker	Stem cell marker	Biomarker diagnostic	Biomarker therapeutic	Biomarker prognostic
Surface m	arkers, CD			
CD24	(332)			
CD44	(333–335) (336)*		(337, 338)	(339)
CD133	(334, 340) (336)*	(340)	(338, 341– 343)	(340, 344)
CD166	(333) (336)*			(333)
Surface m	arkers, not CD			
EpCAM	(335) (336)*		(345, 346) (347)*	
LGR5	(335, 348–350) (336)*	(351)	(352)	(353, 354)
Intracellul	ar markers			
ALDH	(335, 355, 356) (336)*			(355) (357)*
Letm1	(358)			(358)
Nanog	(359, 360) (336)*		(361)	(361, 362)
Oct-3/4	(363, 364) (336)*			(363, 365)
Sall4		(366)		(366)
Sox2	(359, 367, 368)			(367–369)
	(336)*			

**TABLE 5** | Examples of colorectal cancer stem cell markers and their use as diagnostic, predictive, or therapeutic biomarkers.

The table lists examples of cancer stem cell markers and indicates those which have been tested as biomarkers within a therapeutic (metastasis, tumor stage, size, resistance), diagnostic (i.e., resistance), or prognostic (survival, resistance etc.) approach. Starsindicate reviews (\*).

in non-transformed tissues such as epithelial cells (476), and various stem and progenitor cells (477, 478). EpCAM is involved in proliferation and differentiation as well as in cell signaling and formation and maintenance of organ morphology (479). In cancer tissue, EpCAM is homogeneously expressed on the cell surface, while in epithelia it is expressed on the basolateral side (476).

In breast cancers, the expression of EpCAM is correlated to CSC-like phenotypes that promote formation of bone metastases in mice (480). In lung cancers, the expression of EpCAM is often associated with the expression of CD44 and CD166. Triple positive cells show increased clonogenicity, spheroid formation, self-renewal capacity, and show increased resistance to both 5-fluouracil and cisplatin (62).

As one of the first CSC markers, EpCAM has been evaluated as a therapeutic biomarker (compare **Tables 1–5**). Targeting EpCAM with different antibody formats has been performed in colorectal as well as breast cancers (347). In colorectal cancers, a therapeutic approach targeting EpCAM<sup>+</sup> cells with aptamers has been performed in pre-clinical conditions (345, 346).

**TABLE 6** | Examples of AML cancer stem cell markers and their use as diagnostic, predictive, or therapeutic biomarkers.

Marker	Stem cell marker	Biomarker diagnostic	Biomarker therapeutic	Biomarker prognostic
Surface n	narkers, CD			
CD33		(370)	(371–392)	(393)
CD123	(370, 394– 396)	(395, 397– 399)	(373–376, 397, 400–412)	(394, 399, 403, 413)
Surface n	narkers, not C	D		
CLL-1	(414–416)	(370)	(414, 417–419)	(415, 420)
TIM3	(421)		(422)	(420, 423)
Intracellu	lar markers			
ALDH	(424)			(424, 425)
Nanog	(426)	(427)		(426)
Oct-3/4	(428)	(429)		(429–431)
Sox2				(431, 432)

The table lists examples of cancer stem cell markers and indicates those which have been tested as biomarkers within a therapeutic (metastasis, tumor stage, size, resistance), diagnostic (i.e., resistance), or prognostic (survival, resistance etc.) approach.

# Intracellular Biomarkers as Regulators of Stemness in Solid Cancers

Both embryonic and CSCs show unlimited growth, invasive capacity and are characterized by an undifferentiated cellular state (481). This feature depends on transitions between epithelial and mesenchymal states, regulated by a network of intracellular pluripotency transcription factors. As reviewed by Hadjimichael et al. and also described by others pluripotency in ESC is regulated by a core-network of transcription factors, consisting amongst others of Oct-3/4, Sox2, Nanog, Klf4, and c-MYC as well as signaling pathways such as the Jak/Stat, Wnt/ß-catenin, Hedgehog/Notch, TGF-ß as well as FGF signaling pathways (367, 482, 483). The core-pluripotency network consisting of Nanog, Oct-3/4 and Sox2 (described in detail below) activates genes of self-renewal and suppresses genes involved in differentiation (482). Pluripotency factors as well as signaling pathways have been indicated as biomarkers for CSCs as shortly described below (compare Tables 1-5). Of note, the tables do not include all biomarkers, however describe the most abundant ones reported in the literature.

### Sox2

The transcription factor Sox2 belongs to the SRY-related HMGbox (SOX) family, and is involved in the maintenance of an undifferentiated cellular phenotype (367). Its aberrant expression in cancers often leads to increased chemotherapy resistance and asymmetric divisions, as observed in colorectal cancers (368). In those, Sox2 expression correlates with a stem cell state and with a decreased expression of the caudal-related homeobox transcription factor 2 (CDX2), which could serve as a prognostic marker for a poor prognosis (367, 368). In gastric cancers, expression of Sox2 correlates with the tumor stage as well as with a poor prognosis (247, 288). The formation of tumor spheroids *in vitro* also correlates to the overexpression of CD44 and CD133 as well as the transcription factors Sox2, Nanog and Oct-3/4 (247).

Marker	Stem cell marker	Biomarker diagnostic	Biomarker therapeutic	Biomarker prognostic
Surface m	arkers, CD			
CD25	(433–437) (438–440)*	(439)*	(441)	
CD26	(433–437, 442–445) (438–440)*	(443, 446) (439)*	(434, 447, 448)	(443)
CD33	(433, 434) (438–440)*			
CD36	(434, 435) (438)*		(435)	
CD117	(433, 434, 437) (439, 440)*			
CD123	(434, 449–451) (439, 440)*		(449, 450)	
Surface m	arkers, not CD			
IL1RAP	(433–437, 452, 453) (438–440)*	(439)*	(452, 453)	(437)
Intracellul	ar markers			
JAK/STAT	(433) (438)*			
Wnt/β-	(454–456)		(454, 458, 459)	
catenin	(438, 457)*		(457)*	
FOXO	(460) (438)*		(460)	
Hedgehog/	Smo/Gli2 (461) (438)*		(461)	

**TABLE 7** | Examples of CML cancer stem cell markers and their use as diagnostic, predictive, or therapeutic biomarkers.

The table lists examples of cancer stem cell markers and indicates those which have been tested as biomarkers within a therapeutic (metastasis, tumor stage, size, resistance), diagnostic (i.e., resistance), or prognostic (survival, resistance etc.) approach. Stars indicate reviews (\*).

However, in another study, Sox2 levels were downregulated in gastric cancers in comparison to normal tissue and high Sox2 expression correlated with decreased metastasis and a better prognosis for the patient due to increased p21 levels (293). Therefore, the oncogenic functions of Sox2 are controversially discussed in gastric cancers, in which Sox2 might also have tumor-suppressor functions. These different functions seem to depend on the cancer origin and cellular context (484).

### Oct-3/4

Oct-3/4, also known as POU5F1, belongs to the POU homeobox gene family and is also a regulator of pluripotency in mammalian stem cell population. Oct-3/4 is upregulated in several cancers and may support the neoplastic transformation and resistance (485). In colorectal cancer cells, Oct-3/4 causes increased migration and liver metastasis (363, 486) correlating with poor survival (365). As reviewed by Prabavathy et al. Oct-3/4 expression is correlated to increased self-renewal and metastasis in lung cancer cells (67). A meta-analysis showed that Oct-3/4 expression in lung cancer was associated with poor outcomes concerning the differentiation degree, the TNM Classification of Malignant Tumors (TNM) and lymphatic metastasis (136).

In hepatocellular carcinoma (HCC) Oct-3/4 expression was correlating with tumor size and recurrence (309).

### Nanog

Nanog is a homeobox domain transcription factor widely expressed in human cancers (487). In colorectal tumors its expression was significantly increased in CD133<sup>+</sup> cells, and on the basis of a univariate analysis, Nanog expression correlated linearly to liver and lymph node metastasis and the TNM stage. It might therefore be useful as a prognostic biomarker in post-operative liver metastasis (362). In breast cancer, expression of Nanog and Oct-3/4 has been correlated to a poor prognosis of the patient as well as EMT (220, 221). In HCC cell lines Nanog expression drives selfrenewal and invasion, metastasis, and drug resistance (298).

# Biomarkers for CSC Populations in Hematological Cancer

CSC biomarkers of AML and CML have been listed in **Tables 6**, 7. They indicate commonly used markers and point out possible functions of these markers as biomarkers in prognosis, therapy, and diagnosis. Below a short introduction of the most relevant markers is given.

### CD44

As mentioned above, CD44 is a common marker shared by many cancers (60). In hematological cancers, CD44 expression is functionally associated with chemotherapy resistance (488, 489). The expression of CD44 in AML is significantly correlated with a poor overall survival (OS) (490). Furthermore, CD44 was shown to be significantly higher expressed in non-remission AML patients (490). A highly relevant function of CD44 for LSCs is the adhesion to the bone marrow niche (491).

### CD123 and CD33

In hematological malignancies, such as AML, CD123 as well as CD33 have been described as the "classical" CSC markers (492, 493). CD123 is a marker expressed on LSCs (395, 397, 494), but not exclusively (395, 398). In AML patients, CD123 expression correlates to the therapy response rate (413, 495), the relapse risk (403), and a shorter disease-free period and OS (399, 413). CD123 has been associated with increased proliferation and differentiation (494, 496).

CD33 is historically, the most commonly used marker for AML stem cells, with clinical implementation of CD33 targeting, dating back to the Food and Drug Administration (FDA) approval of gemtuzumab ozogamicin (GO) in 2000 (497). CD33 is highly expressed on blasts in around 85–90% of AML patients (433, 438, 497) and also expressed at higher densities in CML (433, 438) but less on healthy hematopoietic stem cells (HSCs). These cells are additionally characterized by expression of CD25, CD26, and Interleukin-1 receptor accessory protein (IL-1RAP) and also other markers (440).

### CLL-1

The C-type lectin-like molecule-1 (CLL-1) is a promising alternative to the "classical" LSC targets (414). The majority of

AML patients shows CLL-1<sup>+</sup> LSCs, a marker not being expressed on HSCs (370, 414-416). Compared to CD33, CLL-1 was not only more frequently and stronger expressed on LSCs, but also not or more weakly expressed on normal tissues leading to reduced off-target effects after treatment with a respective antibodydrug conjugate. Therefore, CLL-1 might be a more suitable and specific LSC target than CD33 (414). A high expression of CLL-1 is associated with poor prognosis (420) and a faster relapse (415) in AML. Interestingly, controversial observations have been made using CLL-1 as a biomarker after chemotherapy. The diagnostic value of CLL-1 is discussed controversially: while Zhang et al. showed that CLL-1 was increased after chemotherapy (371), others showed that there is no difference between CLL-1 expression at diagnosis and at relapse (415) or even detected a decreased CLL-1 expression at relapse (370). The relevance of CLL-1 as a prognostic biomarker for chemotherapy failure or relapse is therefore still unclear. Its expression is not detectable within the chronic phase of CML (440).

### TIM-3

Another "non-classical" LSC biomarker is T-cell immunoglobulin and mucin 3 (TIM-3), that is highly expressed on LSCs but not expressed on healthy HSCs (498). It is correlated to a poor prognosis (420) and treatment failure (423). Stem cell properties of TIM-3<sup>+</sup> cells were confirmed by engraftment in a xenograft mouse model (421).

# Intracellular Biomarkers as Regulators of Stemness in Hematological Cancers

The core-network of pluripotency associated transcription factors as well as signaling pathways have also been analyzed in hematological cancers. Fifty AML patients have been analyzed for the expression of Sox proteins, which are overexpressed in 10-22% of the patients. The analysis showed that high levels of Sox proteins may have a prognostic value (432). The analysis of Oct-3/4 expression correlated with an unfavorable prognosis and is associated with FMS-like tyrosine kinase 3-internal tandem duplications (FLT3-ITD) (430).

Activation of stemness-associated pathways especially in CML has been shown to promote extensive proliferation and has been linked to the onset of blast crisis, which is associated with a loss of differentiation of the leukemia initiating cells. An important impact on this effect has the Wnt/ß-catenin pathway (46) that promotes HSC proliferation, independent of the bone marrow niche (5, 22, 499). Especially, resistance to the tyrosine kinase inhibitor imatinib has been shown to correlate to an increased nuclear localization of ß-catenin (454, 458, 500). Inhibitors targeting the Wnt pathway have been shown to be of advantage for example in long-term cell cultures (500). Additionally, the hedgehog pathway has been suggested to be involved in chemotherapeutic resistance in CML, which is also characteristic for chronic phase CML cells (47). Mouse studies also indicate the involvement of the hedgehog pathway (46, 47), which has been implicated as a therapeutic biomarker for CML (456, 461).

To summarize, CSCs at tumor initiation originate from either differentiated cells or adult tissue resident stem cells (5, 19, 22).

Several data indicate that the origin strongly correlates to the aggressiveness of the tumor. Therefore, extra- and intracellular biomarkers that characterize CSCs have been identified and implemented to be of diagnostic and prognostic advantage. However, stem cells are subject to a high degree of plasticity modulated by the TME (19), that is significantly changed by chemo- and radiotherapies and composed of several different cell types. In the following section, focus will be laying on senescent tumor cells as part of the TME as they have long-term influence on TME and CSC development and progression.

# THE ESCAPE OF CANCER STEM CELLS FROM THERAPY

At the moment first-line therapeutic treatments in progressed tumors include in the most cases surgery, chemo- as well as radiotherapies (501) (compare **Figure 2**). Those have been shown to induce DNA damage and to trigger senescence in cancer cells, a process known as therapy-induced senescence (TIS) (10, 502, 503). TIS will cause a decreased tumor size and attracts immune cells such as neutrophils, monocytes as well as T-cells toward senescent tumor site (503). However, over a long-term period the anti-tumorigenic effects of TIS are lost and the cancer might gain stemness causing tumor relapses (**Figure 2**).

### Therapy-Induced Senescence: Its Hallmarks, Biomarkers, and Its Role in CSC Generation

Agents that induce DNA damage such as chemo- and radiation therapies have been identified to trigger senescence in differentiated cancer cells (10). TIS has been in the research focus, because it significantly contributes to the long-term outcome of patients (12). The DNA damage response ultimately activates one or several tumor suppressors pathways [p53, p16 (Ink4a), p21 (Waf1), and retinoblastoma (RB)], that trigger and maintain the senescence growth arrest (504). However, it is important to mention that the senescence phenotype can also be induced in cancer cells which lack functional p53 and RB protein (504). TIS and senescence in general, are recognized as a double-edged sword, that on the one hand leads to the attraction of immune cells, inflammation, and elimination of senescent tumor cells and correlates with a positive post-treatment prognosis and treatment outcome (505-507). On the other hand, senescence can play a strong pro-tumorigenic role that supports CSC generation, as described below.

Senescent cells are characterized by biochemical and morphological changes such as flattening and/or nuclear enlargement (508). There are several classical biomarkers of cellular senescence and they comprise: senescence-associated beta-galactosidase (SA-ß-gal) activity, expression of p53 protein, the amount of p53 in the nucleus, increase in expression of p14 (Arf), p16 (Ink4a) and p21 (Waf1), SASP, and often senescenceassociated heterochromatic foci (SAHF) (12, 505, 507, 509–515). Furthermore, senescent cells display low Ki67 levels and show levels of heterochromatin protein 1 (HP1) gamma (516), as well



FIGURE 2 | Kinetic of tumor development in pre-, early-, and late-therapeutic period upon application of chemo- and/or radiation therapy: current situation in the clinic. (A) In the pre-therapeutic situation, heterogeneous tumors are composed of several cell types, including CSC, tumor cells, TAMs, and CAFs; all characterized by biomarkers. (B) In the early post-therapeutic period, upon application of the first-line treatment (that currently uses mostly chemo- or radiotherapeutic regimens) several important changes occur in the tumor, in particular: tumor cells or CAFs die due to the therapy or become senescent, whereas CSCs mostly survive the treatment. Senescent cells (tumor cells and CAFs) attract immune cells toward the senescent site via SASP. The SASP therefore plays a positive role and attracts immune cells in this early post-therapeutic situation. Attracted immune cells promote the clearance of dead, of necrotic, and senescent tumor cells and CAFs. (C) In the late post-therapeutic situation uncleared senescent tumor cells and senescent CAFs and SASP thereof play a negative (pro-tumorigenic) role and support tumor development. SASP molecules provide stimulating factors for CSCs for further uncontrolled proliferation as well as their maintenance. Also, remaining senescent tumor cells acquire additional mutations that promote activation of a stemness phenotype and finally a tumor relapse.

as di- or tri-methylated lysine 9 of histone H3 (H3K9Me2/3) and histone H2A variant (macroH2A) (505, 517, 518). The usefulness of telomere length as a biomarker of senescence has been questioned (505).

Biomarkers that underline the effect of a therapeutic approach based on the induction of senescence have to be evaluated carefully and quite often simultaneously. The investigation of senescence markers after post-operative chemotherapy in muscle-invasive bladder cancer (MIBC) revealed that the simultaneous expression of several markers involved in the p53 pathway has to be checked to correctly assess the pathological outcome of MIBC (509). The analysis revealed that the expression of p14 (Arf) was associated with an impaired response to chemotherapy and poor prognosis, whereas p21 (Waf1) expression was related to reduced tumor cell proliferation (509).

TIS can play an anti-cancerous role (503). As demonstrated in our studies in premalignant and malignant liver disease, the induction of senescence leads to a so-called "senescence surveillance" mechanism, which relies on innate and adaptive

immune cells. These cells clear senescent premalignant cells, thereby protecting premalignant liver from cancer development (535, 536). Interestingly, in further studies, we could show that the chemokine (monocyte chemoattractant protein 1, MCP-1) axis is of importance for the induction and maintenance of senescence and for the sufficient immune surveillance in the liver (525). Several biomarkers of senescence were found to correlate with a disease-free survival or with an improved OS in several solid cancers (516, 524). One such indicator, a lysosomalbeta-galactosidase (GLB1) that hydrolyzes beta-galactose from glycoconjugates and represents the origin of SA-ß-gal, was reported as a reliable senescence biomarker in prostate cancer (516). Inhibition of the cyclin-dependent kinase 4/6 (CDK)-RB pathway by a novel drug, SHR6390, resulted in reducing the levels of Ser780-phosphorylated RB protein and correlated with the G1 arrest as well as with cellular senescence in a wide range of human RB<sup>+</sup> tumor cells in vitro (520). Xiang et al. identified seven senescence-associated genes (SAGs, Table 8) significantly decreased in senescent cells and increased in HCC TABLE 8 | Biomarkers of therapy-induced senescence (TIS).

Biomarker	References
Senescence-associated beta-galactosidase (SA-β-Gal)	(12, 14, 504, 505, 510, 516–520)
P53	(14, 504, 520, 521) (507)*
Retinoblastoma (RB) Protein (CDKN2A; Ser780phosphorylated RB protein; cyclin-dependent kinase (CDK) 4/6-retinoblastoma)	(12, 14, 504, 519–521) (507)*
P14 (human)	(12, 509, 510, 514, 515, 519)
P19 (mouse)	(505, 507, 513)*
P16 (INK4A; CDKN2)	(12, 14, 509, 512, 515, 519, 522)
	(505, 507, 513, 514, 516)
P21 (WAF1)	(14, 509, 522) (505, 507, 513)*
Senescence-associated heterochromatic foci (SAHF)	(12, 509, 510, 515, 519) (505, 507, 513)
Heterochromatin protein 1 (HP1) gamma	(509, 516, 518)
Telomere length	(505)*
Di- or tri-methylated lysine 9 of histone H3 (H3K9Me2/3)	(505, 517, 518)
Histone H2A variant (macroH2A)	(505, 517, 518)
Lysosomal-beta-galactosidase (GLB1)	(516)
Inhibition of growth (ING) family of proteins $(ING-1, -2, -3, -4, -5)$	(523)
Senescence-associated genes (SAGs) family: [18B (KIF18B), Citron kinase (CIT), Centrosomal protein 55 (CEP55), minichromosome maintenance complex component 5/7 (MCM), Cell division cycle 45 (CDC45), enhancer of zest homolog 2 (EZH2)]	(524) e
Senescence-associated secretory phenotype (SASP)	(12, 14, 510, 519) (505, 507, 509, 522)
Soluble TNF-receptor-II	(11, 523)
Chemokine (C-C motif) receptor/ligand 2, (CCR2/CCL2); Monocyte chemoattractant protein 1 (MCP-1) axis	(525)
IL-1	(526)
IL-6	(527–531)
IL-8	(528, 531, 532)
Regulated on activation, normal T cell expressed	(526, 527) I (533, 534)
and secreted (RANTES)	

Examples of the most important biomarkers of TIS are listed. Stars indicate reviews (\*).

tissues (524). Interestingly, those SAGs were strongly associated with OS, especially in Asian populations, and had a better predictive accuracy in comparison to serum AFP in predicting OS of HCC patients (524). Recently, Smolle et al. reviewed and underlined the role of members of the inhibition of growth (ING) family. These act as tumor suppressors, regulating cell cycle, apoptosis, and cellular senescence. The authors proposed them as clinically useful biomarkers in the detection and prognosis of lung cancer (523).

In line with the positive role of senescence, evidence exists regarding the role of TIS in turning "cold" tumors

toward a "hot" phenotype that results in activating immune responses against tumor antigens (503). As reported in  $Her2^+$  breast cancer patients treated with Trastuzumab and chemotherapy, the treatment-induced epitope spreading was characterized by increased antibody responses not only to the tumor antigen Her2, but also to endogenous CEA, insulin-like growth factor-binding protein 2 (IGFBP2), and p53 (521).

TIS is a very important protective mechanism that is induced immediately after chemo- or radiation therapy. TIS mediates the recognition and clearance of senescent tumor cells by immune cells (503, 535). Induction of TIS after the therapy is associated with a better prognosis and OS (524). However, if senescent tumor cells are not cleared in a timely fashion, their role at a later time points shifts from positive to negative, as discussed in the section below.

### Negative Role of TIS: Cancer Progression

Several studies report a pro-tumorigenic effect of TIS leading to cancer recurrence and support of tumor development (503). Uncleared senescent cells acquire additional mutations, thereby escaping the cell cycle arrest and transform into malignant cells (536). Moreover, factors secreted by senescent cells are also reported to play a strong tumor-promoting role (526).

Was et al. suggested that senescent human colon cancer cells (HCT116) that appear during a doxorubicin-based therapy enter a "dormant" cellular state, survive the treatment, and cause tumor re-growth (537). Importantly, the recent findings by Scuric et al. suggest a long-term effect of chemotherapy and/or radiation exposure upon TIS (11). In this study, markers of cellular senescence, including higher DNA damage and lower telomerase activity, were observed many years later in breast cancer survivors (11). Elevated levels of a soluble tumor necrosis factor (TNF)-receptor-II, a pro-inflammatory biomarker and one of the main SASP molecules, were also detected (11). A negative effect of SASP was correlated to a p53 single-nucleotide polymorphism (SNP) at codon 72 which is correlated to increased risk of breast cancers (538). Using a humanized mouse model, Gunaratna et al. showed that SASP caused an increased invasion of pro-inflammatory macrophages (522). However, the inflammation proceeded into a chronic inflammation with pro-tumorigenic action and tumor-associated macrophages (TAMs) contributed to angiogenesis and increased tumor growth rates (522). Also, senescent cancer-associated fibroblasts (CAFs) and, in particular, expression of Caveolin-1 (CAV1) promote tumor invasion in pancreatic cancer (539). Moreover, in clinicopathological characteristics of patients, a high CAV1 expression directly correlates with higher levels of serum tumor antigens, with the rate of advanced tumor stage, and with significantly worse outcomes in both overall and disease-free survival (539).

It has been suggested that cancer therapies, especially chemoand radiotherapies, possess long- and late-term pro-tumorigenic side effects and could therefore contribute to the relapse of the malignant disease they were intended to treat (540). Such long-term effects could be caused by the decreased removal of senescent cells, as described below.

### Cancer Stemness: Senescence Escape

As mentioned above, cells undergoing senescence can still escape the senescence program and become malignant while acquiring additional mutations (519, 535, 536) (Figure 2). In our studies, we observed a spontaneous mutation [a deficiency in p19 (Arf)] in Ras-expressing hepatocytes, which resulted in a fullblown HCC development using a Ras-induced precancerous liver disease model (535, 536). The reversibility of TIS can be caused through the inactivation of tumor suppressors p53, p16 (Ink4A), p19 (Ink4d), and/or RB (504, 507, 519). Additionally, the over-expression of CDC2/CDK1 and survivin can promote cancer stem cell survival and can also promote the development of polyploidy (507). In general, mutations in CDKN2A, coding for p16 (Ink4a, CDKN2A), p21 (Waf1, CDKN1A), and p27 (Kip1, CDKN1B) as well as E2F3 and EZH2, and a high c-MYC expression might result in low percentages of senescent cells (504, 519). Moreover, particular mutations completely protect melanoma cells from cell cycle arrest upon chemotherapy: DMBC29 melanoma cells that carried a EZH2<sup>S412C</sup> mutation. expressed c-MYC at a low level and a wild type of CDKN2A did not undergo senescence, in contrast to many melanoma cells treated with vemurafenib and trametinib (519).

An escape of cells from senescence was also identified by Milanovic et al. in B-cell lymphoma studies (14). In those studies, the researchers showed that senescent cells substantially upregulated an adult tissue stem cell signature and activated Wnt signaling (14). This senescence-associated stemness was an unexpected cell-autonomous phenotype that caused the generation of cells with a higher tumorigenic potential *in vitro* (14).

However, escape from senescence is not the only pathway that promotes an increase in the cancer stemness phenotype. Stemness within the tumor tissue is also regulated indirectly by signaling molecules which support the maintenance of stemness in CSCs as well as non-CSCs, as described in the following sections.

### Cancer Stemness: SASP and CSC Maintenance

The stemness phenotype within a tumor can also be mediated via SASP (526). Several studies address the strong pro-tumorigenic phenotype (526) whose cytokines can mediate the maintenance of CSCs. The most prominent interleukins (IL) of SASP are IL-1,-6, and-8 (526). These cytokines can influence the CSC phenotype and functionality and therefore influence the plasticity phenotype of CSCs.

Using breast cancer cell lines, Di et al. showed that an induction of senescence in mesenchymal stem cells by hydrogen peroxide treatment causes an increased secretion of the inflammatory cytokine IL-6, which led to a higher migratory capacity of breast cancer cells *in vitro* as well as in xenotransplants (541). An increase in the aggressive metastatic chemoresistant phenotype upon inflammatory cytokine stimulation such as IL-1ß, IL-6, and RANTES (regulated on activation, normal T cell expressed, and secreted) was also observed by others (533, 534). Our own work indicated that IL-8 blocks differentiation of hepatocellular premalignant cells, a pathway mediated via mammalian target of rapamycin complex 1 (mTORC1) kinase, that causes an increase in chemotherapy resistance (532). An increase in tumorigenicity and EMT of breast cancer cells has been shown to correlate to an increased expression of CD44 or CSC-like properties and be caused by the senescence-associated IL-8 and IL-6 (527–529). Pathways that might be involved in such cellular reprogramming processes are the JAK2/STAT3 signaling pathway (542), the IL-6/STAT3 and NOTCH cross-talk signaling (187, 530) as well NFkB-IL-6 signaling axis, responsible for the generation of CSCs (531). Interestingly, interference with those pathways by aspirin increased chemosensitivity and decreased self-renewal in breast cancer cells (531). In colorectal cancer cells the inflammatory cytokine IL-6 mediates deacetylation, which subsequently activates NANOG transcription and accumulation of stemness phenotypes, correlating with malignant progression and poor prognosis (543).

To summarize, TIS on the one hand has positive effects that eliminates differentiated tumor cells and also causes invasion of immune cells with anti-tumorigenic functions. On the other hand, senescence causes negative effects that are reflected by protumorigenic functions causing CSC development and a gain of cancer stemness (**Figure 2**).

An additional level of complexity is added by the plasticity of CSCs as well as non-CSCs, which also causes increased cancer stemness, resistance, and relapse. Examples are given in the next paragraph.

### Cancer Stemness: Plasticity of CSCs and Non-CSCs

Cancer stemness is not only triggered by senescence escape and acquisition of stemness phenotypes or supported by maintenance of stemness (544) but also by the plasticity of CSCs and non-CSCs, altogether causing tumor relapses after treatment, as described below.

Plasticity is regulated by the TME that is very heterogeneous and consists of CAFs, TAMs, and neutrophils as well as of cancer-associated adipocytes, tumor-infiltrating lymphocytes, and cancer cells with or without stem cell characteristics (545). Therefore, a clear separation between SASP effects and plasticity cannot be made as several direct and also indirect regulatory networks are involved (**Figure 2**).

Mechanistically, plasticity of cells is a characteristic that ensures robust tissue regeneration and homeostasis (546, 547) and describes the phenotypic and molecular changes of tumor cells increasing stemness and reflecting the tumor's ability to self-renew (18, 548). This phenotype is ultimately closely linked to EMT (15, 548). As described, the transition from the epithelial to mesenchymal state is associated with defined regulatory networks, chromatin remodeling and gene expression programs that are specific to the epithelial, mesenchymal or hybrid cellular state (15-18). Plasticity increases the complexity by suggesting that CSCs can switch between different cellular states, characterized by the expression of surface markers as well as transcription factors (18, 56). Examples for this come from the analysis of different tumor cells: Chaffer et al. demonstrated that CD44<sup>low</sup> cells (non-CSCs) can switch to a CD44<sup>high</sup> phenotype (CSCs) resulting in mammosphere formation, a phenotype that could be induced by upregulation of the zinc finger E-box binding homeobox 1 (ZEB1) protein

expression induced by TGF-ß (548), which is a major cytokine of the TME (545). In NSCLC cell lines, two distinct CSC subpopulations have been described by expression of CD133 and the aldehyde dehydrogenase (ALDH) (549). ALDHs compose an enzyme superfamily with metabolic functions. The analysis of its activity is often used to identify CSCs (550, 551). Analyzing CD133 and ALDH activity, Akunuru et al. separated cancer stem/progenitor cells (CD133+, ALDH<sup>high</sup>) from non-CSCs (CD133<sup>-</sup> or ALDH<sup>low</sup>) and showed that non-CSCs can interconvert into CSCs. The latter process is activated by TGFß signaling or signaling by the zinc finger protein SNAI (Snail) transcription factor family. The described process underlines the dynamic plasticity of CSC/non-CSCs cells (549). After TGF-ß treatment, the authors observed an increase in IL-1ß and IL-6 as well as an increase in CD133<sup>+</sup> and ALDH<sup>high</sup> subpopulations (549).

Interferon-ß (IFN-ß) as well as Oncostatin M (OSM), also cytokines within the TME, have been shown to regulate CSC phenotypes (552). Activation of IFN-ß signaling pathways in non-CSCs blocks the expression of CD44 and Snail, which causes a decrease tumor sphere formation and additionally inhibits invasion (552). In contrast, OSM induces a stemness phenotype in non-CSCs (552). One of the major regulators of colorectal tumor plasticity (either CSCs or cancer cells) are the Wnt-ß-catenin and the KRAS/BRAF/ERK pathways, which have been implicated to regulate tumorsphere formation, selfrenewal as well as resistance, as reviewed by Pereira et al. (553) and Zhan et al. (554). Activation of Wnt-signaling increased sphere and clone formation as well as drug resistance (555, 556). Acquisition of stemness was also described by Perekatt et al. using transgenic mice to analyze the function of Wnt-signaling in tumorigenesis and de-differentiation in the gut (28). The authors show that the inactivation of Smad 4, a factor that regulates the differentiation program, promoted the development of adenomas with characteristics of activated Wnt signaling over long-term periods (28). Such Wnt activation can correlate with increased treatment resistance as reviewed by Mohammed et al. (557). Also in gastric cancer, activation of the Wnt pathway causes an increase in CD44 as well as Oct-3/4 expression and correlates with an increased proliferation (558).

As described above, a gain of stemness due to SASP and CSC maintenance or by plasticity of CSCs and non-CSCs, can cause increased resistance (**Figure 2**). CSCs (pre-existing or post-therapeutically generated *de novo*) can escape the treatment by the expression of drug exporters and detoxification proteins, entrance into dormancy as well as resistance to DNA damage induced cell death (4, 15, 185, 559, 560). Their survival causes tumor relapses (**Figure 2**). To interfere with the relapse, several strategies have been under investigation to block CSC resistance and growth (9, 13), as described below (**Figures 3, 4**).

# ERADICATION OF CSCs: NEW TARGETED APPROACHES

Targeting CSCs has been in the focus of research for many years (13). As reviewed by Shibata and Hoque, the combination of CSC-targeted therapies and conventional non-targeted therapies

can result in a decreased chemoresistance (9). Approaches of CSC-targeted therapies include kinase inhibitors as well as targeting stem cell associated pathways such as Wnt and  $\beta$ -catenin, some of which have already entered the clinical phase (9, 13). Immunological approaches that target CSCs via MHC-restricted killing include adoptive cell transfer, targeting checkpoint inhibitors as well as antibody-based approaches and vaccination. MHC-unrestricted killing based on NK-,  $\gamma\delta$ T-, and chimeric antigen receptor (CAR) T-cell approaches have been established (561, 562). Currently, these approaches are performed after failures of the first-line therapies.

Based on the promising results of CAR T-cellular therapy in treating hematological diseases, CAR T-cell-based approaches have also moved forward into the therapy of solid cancers (563, 564). Although, CAR T-cell-based approaches face difficulties in treating solid cancers, their therapeutic use could be a promising alternative (563, 564).

# **CAR THERAPIES TARGETING CSCs**

## Targeting CD133<sup>+</sup> CSCs

Targeting CD133<sup>+</sup> CSCs in solid cancers has shown quite promising preclinical results either using monotherapeutic approaches (565, 566) or using combinational approaches together with cytostatic agents (567). Recently, a clinical trial testing CD133-directed CAR T-cells in patients with ALL, AML, breast, brain, liver, pancreatic and ovarian cancers as well as colorectal cancers has been completed (NCT02541370, **Table 9**). Initial results showed feasibility, safety, and efficacy of CD133directed CAR T-cells in patients. Especially, HCC patients who were not responsive to sorafenib showed a median progressionfree survival of 7 months (568). In all patients the duration of response ranged from 9 to 63 weeks; three patients showed a continued response at the time of publication. Stable disease was observed in 14 out of 23 patients for 9 weeks to 15.7 months and 21 patients did not show detectable signs of metastasis (568).

Additional studies (**Table 9**) are ongoing for the treatment of relapsed or refractory AML (NCT03473457), relapsed or late staged sarcoma (NCT03356782), as well as glioma (NCT03423992). A case study of a patient receiving CD133directed CAR T-cells after previous chemo- and radiotherapy as well as EGFR-directed CAR T-cell therapy reported a partial response for a period of 4.5 months (569). However, severe toxicities affecting the skin, the oral mucosa, and the gastrointestinal tract were reported (569).

## Targeting CD44<sup>+</sup> CSCs

Although CD44 is a very prominent CSC antigen, only few CAR-based approaches targeting CD44 have been developed. Early approaches that entered clinical trials included monoclonal antibodies and antibody-conjugates. First studies involving <sup>186</sup>Re-conjugated antibody against the splice variant CD44v6 showed advantageous effects at first, however a long-term stable disease was only observed in one patient (570, 571). Likewise, the CD44-directed monoclonal antibody RG7356 showed only modest success in clinical trials with AML patients (572) and solid tumors (468). Tijink et al. coupled the CD44v6-directed antibody bivatuzumab to the cytotoxic antimicrotubule agent



mertansine to produce an antibody-prodrug conjugate (573). Bivatuzumab mertansine was administered to seven patients and two of them showed stable disease during the therapy phase. However, one patient with squamous cell carcinoma of the esophagus died after treatment due to toxic epidermal necrolysis, which caused the premature cancelation of this trial (573). Because of this fatality, two clinical trials that were conducted in parallel for patients with metastatic breast cancer (574) and head and neck squamous cell carcinoma (575) had to be terminated.

Still, there are some promising approaches involving CD44v6directed CAR therapies. For instance, cytokine-induced killer (CIK) cells carrying a CAR against CD44v6 showed anti-cancer effects against sarcoma *in vitro* and *in vivo* (576). Furthermore, a phase I/IIa clinical trial using CD44v6-directed CAR T-cells for AML and multiple myeloma patients is currently recruiting (NCT04097301) (**Table 9**).

## Targeting EpCAM<sup>+</sup> CSCs

Pre-clinical as well as clinical studies targeting EpCAM<sup>+</sup> cancer cells using monoclonal antibodies or CAR constructs have been performed to date using co-culture and xenograft approaches (577–579) (**Table 9**). Combination therapy of EpCAM-directed CAR NK-92-cells and regorafenib, a potent multikinase inhibitor, resulted in a synergistic antitumor effect using for example colorectal cancer cells or xenograft models (580). CAR T-cells targeting EpCAM have been shown to significantly block tumor

growth in xenografts and to secrete cytotoxic cytokines, including interferon- $\gamma$  (IFN- $\gamma$ ) and tumor necrosis factor alpha (TNF- $\alpha$ ) *in vitro* (579). Additionally, an injection of EpCAM-directed CAR T-cells led to delayed disease progression in immunodeficient mice with peritoneal ovarian and colorectal xenografts (581). Currently, there are several clinical trials with EpCAM-directed CAR T-cells listed for patients with various malignancies: three trials are ongoing (NCT02915445, NCT03563326, and NCT03013712), one trial is not yet recruiting (NCT04151186), and four trials are listed with unknown status (NCT02725125, NCT02728882, NCT02735291, and NCT02729493) (**Table 9**).

# LSC-Directed CAR Therapies

In the field of CAR therapeutics, CD123 and CD33 are frequent targets for AML-specific CAR cells (**Table 9**). CAR Tand CAR NK-92-cells redirected against CD33 have entered clinical trials (**Table 9**). Case reports show a good tolerability of CD33-directed CAR NK-92-cells (372), but disease progression after treatment with CD33-directed CAR T-cells was still present (387). Currently, numerous clinical trials using CAR T-cells targeting CD123 are ongoing. NCT03672851 with two participants had to be terminated due to adverse effects (582). Furthermore, first studies implement CLL-1 as a target of CAR T-cells [**Table 9**; (419), NCT04010877 and NCT03222674].



## NEXT GENERATION CARS AND TARGETING OF CSCs IN COMBINATIONAL THERAPIES

For the more efficient CSC elimination, different approaches that have been developed can be used, i.e., tandem CAR Tcells (TanCAR) (583) as well as single universal (U) tricistronic transgene CAR T-cells (UCAR T-cells) (584). Multi-targeting of Her2, IL-13 receptor subunit alpha-2 (IL13Ra2), and ephrin-A2 (EphA2) was shown to overcome antigenic heterogeneity in 15 primary GBM samples and to increase the therapeutic success using xenograft models (584). Targeting two or more antigens may increase the risk for on-target/off-tumor toxicity, since most of the antigens are not only expressed on malignant cells, but also on healthy cells (60, 585). Improved safety, specificity, and flexibility can be obtained using universal CARs (UniCAR) or split, universal and programmable (SUPRA) CARs (585-589). Both consist of an inert and universal CAR construct without a single chain variable fragment (scFv) adaptor molecule domain in combination with a multiple tumor-targeting scFv adaptor molecule (585, 588, 589). In both cases, the activity of CAR T-cells can be regulated by the dosage of the scFv adaptor molecules or by introducing competitive molecules, such as leucine zippers as a regulator for the SUPRA CARs (588, 589). Additional safety of CAR T-cells can be achieved by the induction of suicide genes, e.g., iCasp9 (590, 591) or by inhibitory CAR (iCAR) constructs, in which signaling domains consist of an immuno-inhibitory receptor [e.g., CTLA-4 or PD-1; (592)]. An antigen only expressed on the surface of healthy cells is a target of iCAR and therefore the the attack of non-tumorigenic cells is greatly reduced (592). Specificity can be improved by using synthetic Notch (synNotch) receptors. The binding of synNotch specific to the antigen induces the cleavage of an intracellular domain and activates in turn the transcription of a second CAR, specific to another tumor antigen (593).

To enhance the targeting of solid tumors using CARbased approaches, the combination treatment with conventional chemotherapeutic drugs could be a novel strategy to enhance antitumor response. To test this approach, NK-92 cells were modified with an EGFR-directed CAR construct against renal cell carcinoma (RCC) cell lines (594). In combination with the multikinase inhibitor cabozantinib, EGFR-directed CAR NK-92 cells showed synergistic effects *in vitro* and *in vivo* (594). Cabozantinib also caused a decrease of the antiinflammatory PD-L1 surface expression in renal cell carcinoma cell lines (594). Furthermore, cabozantinib is known to reduce tumor infiltration of immuno-modulatory subpopulations like regulatory T-cells (Tregs) and myeloid-derived suppressor cells (MDSCs) (594, 595). TABLE 9 | Overview of clinical trials using current CAR-cell-based approaches in solid and hematological cancers targeting CSC.

Phase	ID number	Approach	Target	Cell-based therapy	Condition
I	NCT03423992	CAR T	CD133, EGFRvIII, IL13RvIII2, Her-2,EphA2, GD2,	Autologous CAR T-cells	Recurrent malignant glioma
I	NCT03563326	CAR T	EpCAM	WCH-GC-CAR T	Neoplasm, stomach metastases
I	NCT02915445	CAR T	EpCAM	CAR T-cells	Malignant neoplasm of nasopharynx TNM stagingdistant metastasis (M), Breast cancer recurrent
Ι	NCT03766126	CAR T	CD123	Autologous CAR T-cells	Relapsed/refractory AML
I	NCT03672851	CAR T	CD123	Autologous CAR T-cells	Relapsed/refractory AML
I	NCT03190278	UCAR T	CD123	Allogeneic CAR T-cells	Relapsed/refractory AML
I	NCT04106076	UCAR T	CD123	Allogeneic CAR T-cells	Newly diagnosed AML
I	NCT02159495	CAR T	CD123	Autologous/allogeneic CAR T-cells	AML (various) or blastic plasmacytoid dendritic cell neoplasms
Ι	NCT03585517	CAR T	CD123	CAR T-cells	Relapsed/refractory AML
I	NCT04014881	CAR T	CD123	CAR T-cells	Relapsed/refractory AML
I	NCT03114670	CAR T	CD123	Donor-derived CAR T-cells	Recurred AML after allogeneic hematopoetic stem cell transplantation
Ι	NCT03796390	CAR T	CD123	Autologous CAR T-cells	Relapsed/refractory AML
Ι	NCT03126864	CAR T	CD33	Autologous CAR T-cells	Relapsed/refractory AML
I	NCT03795779	cCAR T	CLL1-CD33	CAR T-cells	Relapsed and/or refractory, high risk hematologic malignancies
I	NCT02799680	CAR T	CD33	Allogeneic CAR T-cells	Relapsed/refractory AML
1/11	NCT04097301	CAR T	CD44v6	Autologous CAR T- cells	AML, multiple myeloma
1/11	NCT02541370	CAR T	CD133	Autologous or donor-derived T-cells	Liver cancer, pancreatic cancer, brain tumor, breast cancer, ovarian tumor, colorectal cancer, acute myeloid, and lymphoid leukemias
1/11	NCT03356782	CAR T	CD133	Autologous CAR T cells	Sarcoma, osteoid sarcoma, ewing sarcoma
1/11	NCT03013712	CAR T	EpCAM	Autologous CAR T-cells	Colon cancer; esophageal carcinoma; pancreatic, prostate cancer; gastric cancer, hepatic carcinoma
1/11	NCT03556982	CAR T	CD123	Autologous/allogeneic CAR T-cells	Relapsed/refractory AML
1/11	NCT03222674	Multi-CAR T	CD33, CD38, CD123, CD56, Mucl, CLL-1	Autologous CAR T-cells	Relapsed/refractory AML
1/11	NCT04010877	Multiple CAR T	CLL-1, CD33, and/or CD123	Autologous/allogeneic CAR T-cells	AML
1/11	NCT04109482	CAR T	CD123	Autologous CAR T-cells	Relapsed or refractory blastic plasmacytoid dendritic cell neoplasm, acute myeloid leukemia, and high risk myelodysplastic syndrome
1/11	NCT02944162	CAR NK	CD33	NK-92-cells	Relapsed/refractory AML
1/11	NCT01864902	CAR T	CD33	Autologous or donor-derived T-cells	Relapsed/refractory AML
1/11	NCT03971799	CAR T	CD33	CAR T-cells	Children and adolescents/young adults (AYAs) with relapsed/refractory acute myeloid leukemia (AML)
11/111	NCT03631576	CAR T	CD123/CLL-1	CAR T-cells	Relapsed/refractory AML
-	NCT03473457	Single or double CAR T	CD33,CD38, CD56, CD123, CD117, CD133,CD34, or Mucl	CAR T-cells	Relapsed/refractory AML
II	NCT02729493	CAR T	EpCAM	Autologous CAR T-cells	Relapsed or refractory liver cancer
II	NCT02725125	CAR T	EpCAM	Autologous CAR T-cells	Relapsed or refractory stomach cancer
N.A.	NCT04151186	CAR T	EpCAM,TM4SF1	CAR T-cells	Solid tumor

Source: http://clinicaltrials.gov/.

The combination of the multikinase inhibitor sunitinib and CAR T -cells targeting carbonic anhydrase IX (CAIX) has been shown to be of advantage as sunitinib reduces immunosuppressive components of the TME (596). Improvements could also be made using Her2-directed CAR NK-92-cells (92/5.137.z) in combination with apatinib (597). Treatment with CAR NK-92 alone resulted in an efficient elimination of small Her2<sup>+</sup> tumor xenografts *in vivo*, but not in an elimination of larger solid tumors in gastric cancers (597). A combinatorial treatment with apatinib increased CAR NK-92 cell infiltration into these larger tumor xenografts and resulted in an enhanced antitumor efficacy of the cells (597).

In AML, early approaches focused on the targeting of single markers; combinatorial therapies, targeting more than one marker, have been tested here as well (598). Haubner et al. analyzed optimal combinations of different LSC markers and concluded that CD33/TIM-3 or CLL-1/TIM-3 combinatorial targeting is most suitable since these markers maximally cover AML cells and are minimally co-expressed on HSCs (370). Interestingly, the combination of CD33 and CD123 was found unsuitable (370). Approaches that already implement combinatorial targeting of AML LSCs include tri-specific killer engagers against CD33 and CD123 (374) or CLL-1 and CD33 (i.e., NCT03795779), universal CAR T-cells against CD33 and CD123 (375), and CAR CIK-cells against CD33 and CD123 (376).

## **FUTURE PERSPECTIVES**

Studies obtained in the last 5–10 years confirmed the importance and the urgent need of diagnostic screening of the TME not only before the treatment, but also at several stages in the post-therapeutic period. This is within the context of personalized therapies that are based on the idea to identify the best therapeutic approach for the patient. This approach should be based on the tumors molecular signature, involving the TME. The best and the most appropriate therapeutic options, which match each individual patient's requirements will increase the therapeutic efficacy and will cause fewer side effects.

The particular value of post-therapeutic local biopsies is that they enable the evaluation of tumor relapse risk on the basis of multivariate biomarkers and also provide information on therapeutically addressable targets within the remaining tumor tissue. In-time detection of tumor-promoting cells, which reemerge in the post-therapeutic period (**Figure 3**), will allow an application of the individualized and precise second-line therapy in a timely fashion. Detection of tumor cells with stemness phenotypes will allow for their directed and specific targeting using the second-line treatments, depending on a different mode of action (4, 560). This secondary specific therapy can include, targeted therapies such as e.g., immunotherapies, CAR NK-, and CAR T-cells that mediate a precise eradication of several types of cells: CSCs, CAFs, and/or remaining senescent cells. To increase the specificity and therapeutic outcome and to decrease severe side effects, CAR-based therapeutics are constantly being optimized, as discussed in the section above. Special needs are: improvement of target specificity in combination with decreased off-target effects. In addition, secondary therapies could also include senolytic drugs that selectively kill senescent cells as it was discussed in a recent comprehensive review by Short et al. (599). These therapies cause very low or minor side-effects after their administration (599). In the post-therapeutic period, however, it is important to focus on the biomarkers of CSCs as well as the biomarkers of senescent tumor cells, tumor-promoting SASP molecules, CAFs and TAMs. These cells and molecules strongly influence tumor relapse and their monitoring and their in-time elimination is crucial (Figure 3). As currently available blood test systems are not sensitive enough to detect local changes in the TME, other methods for instance local biopsies and subsequent multivariant analysis of obtained tissues should be used whenever possible and even after many years upon the first-line therapy (Figure 3).

The analysis of multivariant biomarker, however is not only of importance within the post-therapeutic situation. A detailed understanding of the tumor composition before the treatment could allow straight forward first line therapies (**Figure 4**). Target analysis includes CSCs, CAFs, tumors cells and TAMs, and other tumor-promoting cells. Therapeutic options such as chemotherapy and radiotherapy in combination with small molecules and immunotherapies (CAR cells) could tremendously improve the outcome of the first-line approaches and predict relapses (**Figure 4**). Combinations already in the first-line therapy are especially required in advanced stages of malignant disease.

In conclusion, our review gives an overview of the most important biomarkers of CSCs in the TME. Furthermore, we underline the value of local biopsies and precise diagnostics and screening of biomarkers in both pre- and post-therapeutic situations (**Figures 3**, **4**). We suggest the implementation of those strategies in the first and second-line personalized therapy required to eradicate the remaining tumor-promoting senescent tumor cells, CAFs, TAMs, and finally CSCs to protect from tumor recurrence.

The high costs are one point of contention regarding the biopsies and their analysis as well as the implementation of immunotherapies into the first and secondary line targeted therapies. However, considering the costs for therapies, comprising resection, and medication strategies, as well as the patient's sufferings due to a re-emerged fullblown cancer, the targeted therapy will help to save the patients and clinics from high personnel, emotional, and medicinal costs.

# **AUTHOR CONTRIBUTIONS**

SF, UK-B, and TY performed a conceptualization for the review and defined the future perspectives. LW, A-KK, HS, RK, SD, AS, A-RB, TY, SF, and UK-B analyzed the publications and created the figures and tables. All authors contributed to the article and approved the submitted version.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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